PATENT
Docket No. 235.00010101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	ALTMAN, Elliot	Group Art Unit:	1653
Serial No.:	09/701,947	Examiner:	LIU, Samuel W.
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For: STABILIZED BIOACTIVE PEPTIDES AND METHODS OF IDENTIFICATION, SYNTHESIS AND USE

DECLARATION UNDER 37 C.F.R. §1.131

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Elliot Altman, Ph.D., declare and say as follows:

1. I am the sole inventor of the subject matter of claims 89-97, 104, 105 and 120-128 presently pending in the above-identified U.S. Patent Application Serial No. 09/701,947, filed on December 5, 2000.

2. I received a Ph.D. in Biology in June 1991, from the California Institute of Technology; a B.S. in Chemistry (Magna Cum Laude) in June 1980, from Texas A&M University and a B.S. in Zoology (Magna Cum Laude) in December 1979, from Texas A&M University.

3. From 1990-1994 I worked as a Postdoctoral Fellow in the Department of Biology at the University of Utah. Since 1995 I have been employed by The University of Georgia, first as an Assistant Professor in the Department of Biology (1995-1999) and since 1999 as the Director of the Center for Molecular BioEngineering. My research activities presently focus on cellular

metabolic engineering, structural biochemistry and drug discovery using molecular genetics. I have published over 20 papers on these and related topics.

4. The instant application is a 35 U.S.C. §371 application of PCT/US99/23731, filed on December 5, 2000 (International Filing Date of October 12, 1999), which claims the benefit of U.S. Provisional Patent Application Serial No. 60/104,013, filed October 13, 1998, and also to U.S. Provisional Patent Application Serial No. 60/112,150, filed December 14, 1998.

5. I have reviewed the above-identified application, as well as the cited U.S. Patent No. 6,562,617 (Anderson et al.), and the Response filed herewith. I make this declaration in support of the patentability of the claims of the above-identified application.

6. Prior to October 8, 1998, I conceived of and reduced to practice a non-naturally occurring polypeptide characterized by a bioactive peptide stabilized by a stabilizing group attached to either or both of the N-terminus or the C-terminus of the bioactive peptide. This invention is evidenced by a draft manuscript, which I wrote prior to October 8, 1998, entitled "Stabilizing synthetically engineered inhibitor peptides" attached hereto as Exhibit A.

7. Exhibit A summarizes, at page 2, certain of my investigations carried out prior to October 8, 1998, into approaches that could be taken to circumvent the problem of peptide degradation by modifying the peptides through the use of a stabilizing motif (page 2, last line). Page 2 describes, for example, a synthetic, inhibitory peptide that is fused at either the amino or carboxy terminal to the Rop protein. Pages 3-4 describe an example of an inhibitory peptide protected at its carboxy terminal end due to fusion to the amino terminal end of the Rop protein, and page 4 describes an example of inhibitory peptide that is protected at its amino terminal end due to fusion to the carboxy terminal end of the Rop protein.

8. Page 2 of Exhibit A describes a peptide, e.g. a peptide drug, stabilized by adding two proline residues at both the amino and carboxy terminal of the synthetic peptide.

9. Tables 3 and 4 of Exhibit A further describe stabilized bioactive peptides conceived of and reduced to practice prior to October 8, 1998, namely peptide inhibitors stabilized by two proline residues at both the amino and carboxy terminal ends of the peptide, inhibitor peptides protected at the C-terminal end via the Rop protein, and inhibitor peptides protected at the N-terminal end via the Rop protein.

10. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

3/4/2004 By: Elliot Altman

Date Elliot Altman, Ph.D.

EXHIBIT A

Stabilizing synthetically engineered inhibitor peptides

J. Warren, J. S. Walker, and E. Altman

Department of Microbiology, University of Georgia, Athens, Georgia 30602-2605

To whom correspondence should be addressed

Elliot Altman
Department of Microbiology
527 BioScience Building
University of Georgia
Athens, Georgia 30602-2605
706-542-2900 (Office)
706-542-2674 (FAX)
Altman@bscr.uga.edu (Email)

ABSTRACT

While the use of synthetically derived novel inhibitor peptides as a source of new therapeutics for medicine remains incredibly promising, there is a major problem with implementing this technology, since many synthetic peptides have proven to be unstable and are degraded by peptidases in the host cell. In this study we have investigated methods by which peptides can be stabilized in order to prevent the action of peptidases. Using an *in vivo* approach that our laboratory has developed to screen for synthetic peptides which can inhibit the growth of *Escherichia coli* bacteria, we have found that protecting the amino or carboxy terminal of the peptides via fusion to the very stable Rop protein increases the frequency at which potent inhibitor peptides can be isolated. We have also shown that peptides can be stabilized by adding two proline residues at both the amino and carboxy terminal of the synthetic peptide. Because peptidases appear to function very similarly in all cells, what we have learned in our studies with generating stable peptides that can inhibit the growth of *E. coli* bacteria should be directly applicable to generating peptide drugs which are resistant to degradation by peptidases in human cells.

INTRODUCTION

Over the past several years there has been an increasing interest in using novel synthetic peptides as a means to generate new therapeutic agents for the pharmaceutical industry (9, 11, 14). The premise behind this new area of research stems from what has been learned from studying naturally occurring peptides which possess biological activities. These bioactive peptides for the large part appear to act by binding to a specific protein target (19) and in many cases it has been shown that the peptides inactivate the protein target with incredible specificity. Binding constants for the naturally occurring bioactive peptides that have been studied tend to be in the nM range (18, 21) with binding constants as high as 10^{-12}M having been reported (4, 13).

Two major strategies have been employed by researchers that have been trying to engineer novel synthetic inhibitor peptides. The first strategy involves the use of chemically synthesized combinatorial peptide libraries of up to 10 amino acids in length (9, 11, 12). The second strategy involves the use of fusion phage libraries where randomly encoded peptides that can be much longer in length are fused to a coat protein of a Ff phage which allows the randomized peptide to be displayed on the outside surface of the phage (14, 20). The libraries that are generated via either chemical synthesis or as fusion phage are then usually mixed with a matrix bound protein target and peptides that can bind tightly to the protein target are selected. New peptides are generated, either based on individual peptide sequence data or collective consensus data from multiple peptides, and tested for their inhibitory potential. Peptides are initially tested *in vitro* by measuring their ability to inhibit the enzymatic activity of the protein target and then promising peptides are tested further in clinical trials.

Although there is enormous potential for the development of synthetic inhibitor peptides using the approaches described above, this technology has not become a mainstay in the pharmaceutical industry due to the problem of peptide stability and the unwanted degradation of the potential peptide drug by peptidases in the host cells (3, 8, 26). Approaches to solve this problem have centered on the use of D-amino acids or modified amino acids as opposed to the naturally occurring L-amino acids, cyclized peptides, as well as the development of enhanced delivery systems which would protect the peptides from premature degradation. We wanted to investigate whether approaches could be taken to circumvent the problem of peptide degradation by modifying the peptides through the use of a stabilizing motif.

There are three major classes of peptidases which can degrade larger peptides, the amino and carboxy exopeptidases which act at either the amino or the carboxy terminal end of the peptide, respectively, and the endopeptidases which act on the internal peptide. Aminopeptidases, carboxypeptidases, and endopeptidaess have been identified in both prokaryotic and eukaryotic cells (2, 5, 15) and where they have been extensively characterized, many of these peptidases have been found to function similarly in both cell types (15, 17). Interestingly, in both prokaryotic and eukaryotic systems, a lot more aminopeptidases than carboxypeptidases have been identified to date.

Our laboratory has developed an *in vivo* approach by which novel synthetic bioactive peptides can be identified that inhibit the growth of *Escherichia coli* bacteria (24). In this study, we have used this system to investigate whether inhibitor peptides can be stabilized at either the amino or carboxy terminal ends in order to prevent the action of either amino or carboxypeptidases. Because the action of peptidases appear to be quite similar in both prokaryotic and eukaryotic cells what we have learned in our studies should be directly applicable to designing more effective inhibitor peptides for use as novel drugs.

MATERIALS AND METHODS

Media. Rich LB and minimal M9 media used in this study was prepared as described by Miller (16). Ampicillin was used in rich media at a final concentration of 100 ug/ml and in minimal media at a final concentration of 50 ug/ml. Isopropyl β-D-thiogalactoside (IPTG) was added to media at a final concentration of 1 mM.

Bacterial Strains and Plasmids. ALS225 which is MC1061/F'*lacI*^{q1}*Z+Y+A+* was the *E. coli* bacterial strain used in this study (25). The genotype for MC1061 is *araD139 Δ(araABOIC-leu)7679 Δ(lac)X74 galU galK rpsL hsr- hsm+* (6). pLAC11 is the highly regulable expression vector that was used to make the p-Rop(C) and p(N)Rop- fusion vectors as well as the randomized peptide libraries which were protected by two proline residues at both the amino and carboxy terminals (25).

Construction of the p-Rop(C) fusion vector: The forward primer 5' TAC TAT AGA TCT ATG ACC AAA CAG GAA AAA ACC GCC 3' and the reverse primer 5' TAT ACG TAT TCA GTT GCT CAC ATG TTC TTT CCT GCG 3'were used to PCR amplify a 558 bp DNA fragment using pBR322 as a template. This fragment contained a Bgl II restrction site which was incorporated into the forward primer followed by an ATG start codon and the Rop coding region. The fragment extended beyond the Rop stop codon through the Afl III restrction site in pBR322. The amplified dsDNA was gel isolated, restricted with Bgl II and Afl III, and then ligated into the pLAC11 expression vector which had been digested with the same two restriction enzymes. The resulting p-Rop(C) fusion vector is 2623 bp in size.

Construction of the p(N)Rop- fusion vector: The forward primer 5' AAT TCA TAC TAT AGA TCT ATG ACC AAA CAG GAA AAA ACC GC 3' and the reverse primer 5' TAT ATA ATA CAT GTC AGA ATT CGA GGT TTT CAC CGT CAT CAC 3'were used to PCR amplify a 201 bp DNA fragment using pBR322 as a template. This fragment contained a Bgl II restrction site which was incorporated into the forward primer followed by an ATG start codon and the Rop coding region. The reverse primer pLACed an EcoR I restriction site just before the Rop TGA stop codon and an Afl III restrction site immediately after the Rop TGA stop codon. The amplified dsDNA was gel isolated, restricted with Bgl II and Afl III, and then

ligated into the pLAC11 expression vector which had been digested with the same two restriction enzymes. The resulting p(N)Rop- fusion vector is 2262 bp in size.

Generating the randomized peptide libraries: All of the peptide libraries used in this study were constructed as described by Walker et. al. (24). To construct the randomized peptide libraries for use with the p-Rop(C) fusion vector, the oligonucleotides 5' TAC TAT AGA TCT ATG $(XXX)_{20}$ CAT AGA TCT GCG TGC TGT GAT 3', and 5' ATC ACA GCA CGC AGA TCT ATG 3' were used. After extension the resulting dsDNA was digested with Bgl II and ligated into the pLAC11 expression vector which had been digested with the same restriction enzyme and subsequently dephophorylated using alkaline phosphatase. To construct the randomized peptide libraries for use with the p(N)Rop- fusion vector, the oligonucleotides 5' TAC TAT GAA TTC $(XXX)_{20}$ GAA TTC TGC CAC CAC TAC TAT 3', and 5' ATA GTA GTG GTG GCA GAA TTC 3' were used. After extension the resulting dsDNA was digested with Bgl II and ligated into the pLAC11 expression vector which had been digested with the same restriction enzyme and subsequently dephophorylated using alkaline phosphatase. To construct randomized 20 amino acid peptide librariess which contained two proline residues at the amino and carboxy terminal ends of the peptides, the oligonucleotides 5' TAC TAT AGA TCT ATG CCG CCG $(XXX)_{16}$ CCG CCG TAA TAA GAA TTC GTA CAT 3' and 5' ATG TAC GAA TTC TTA TTA CGG CGG 3' were used. After extension the resulting dsDNA was digested with Bgl II and EcoR I and ligated into the pLAC11 expression vector which had been digested with the same two restriction enzymes. In the randomized oligonucleotides a X denotes that an equimolar mixture of the nucleotides A, C, G, or T was used.

Chemicals and Reagents. Extension reactions were carried out using Klenow from New England Biolabs while ligation reactions were performed using T4 DNA ligase from Life Sciences. Alkaline phosphatase (calf intestinal mucosa) from Pharmacia was used for dephosphorylation. IPTG was obtained from Diagnostic Chemicals Limited.

RESULTS

Isolation and characterization of inhibitor peptides that are protected at their carboxy terminal end due to fusion to the amino terminal end of the Rop protein: In our initial studies with inhibitor peptides (24), we had used a completely randomized oligonucleotide library in order to direct the synthesis of up to 20 amino acid peptides in the highly-regulable expression vector, pLAC11. In a screen of 20,000 peptides, we found 21 which could inhibit the growth of *E. coli* bacteria. While most of the inhibitors encoded up to 20 amino acid peptides as expected, two of the most potent inhibitors turned out to be fusion peptides in which the carboxy-terminus of the peptide had become fused to the amino-terminal end of the Rop protein (the rop gene is located downstream from where the oligonucleotides are inserted into the pLAC11 expression vector). Because the Rop protein is known to form a very stable structure (7), we deduced that the Rop protein was likely serving as a stable protein anchor which protected the carboxy terminal end of the two inhibitor peptides. Rop is a small 63 amino acid protein that consists of two antiparallel a-helices connected by a sharp hairpin loop. It is a dispensable part of the ColE1 replicon which is used by plasmids such as pBr322 and it can be deleted without causing any ill-effects on the replication, partitioning, or copy numbers of plasmids that contain a ColE1 ori (22).

To test whether peptides could be stabilized by fusing the carboxy terminus of the peptides to the amino terminus of the Rop protein, the p-Rop(C) fusion vector shown in Figure 1, which is a derivative of pLAC11, was constructed as described in Materials and Methods. In order to isolate potential inhibitor

peptides which were protected at their carboxy terminal end, a totally randomized oligonucleotide library that encoded up to 20 amino acid peptides was cloned into the p-Rop(C) fusion vector as shown in Figure 2 and transformed into *E. coli* under repressed conditions. In our initial studies of 20,000 anchorless peptides, only one turned out to be a potent inhibitor which could inhibit the growth of *E. coli* for two days on plates. We used this frequency as a barometer by which to judge whether protecting the carboxy terminus of peptides with the Rop protein would increase the number of inhibitor peptides that could be isolated.

10,000 peptides which were protected at their carboxy terminal end were screened using our previously described grid-patching technique and 16 two day inhibitors were isolated. Unlike our original anchorless inhibitors that we characterized which were only inhibitory on minimal media, we found that many of the Rop fusion inhibitors were also inhibitory on rich media as well. To verify that all of the inhibitors were legitimate, we made plasmid DNA from each inhibitory clone, transformed them into a fresh background, and then checked that they were still inhibitory on plates and that their inhibition was dependent on the presence of the inducer, IPTG. In order to make a more accurate assessment of how inhibitory the inhibitors were, we subjected the first ten inhibitors to a growth rate analysis in liquid media. To do this minimal and rich cultures containing either the inhibitor to be tested or p-Rop(C) as a control were diluted 1 to 100 into new media and induced with 1 mM IPTG. OD_{550} readings were then taken hourly until the cultures had passed log phase. Growth rates were determined as the spectrophotometric change in OD_{550} per unit time within the log phase of growth. The inhibition of the growth rate was then calculated for the inhibitors using p-Rop(C) as a control. As indicated in Table 1, all of the inhibitors inhibited bacterial growth at levels greater than 90% in minimal media and most of the inhibitors inhibited bacterial growth at levels greater than 80% in rich media.

Isolation and characterization of inhibitor peptides that are protected at their amino terminal end due to fusion to the carboxy terminal end of the Rop protein: Because of the success that we had isolating potent inhibitor peptides which were protected at their carboxy terminal end by the Rop protein, we also wanted to test whether protecting the amino terminal end of the peptides would increase the frequency of potent day two inhibitors that could be isolated. Additionally, there are many more known aminopeptidases than carboxypeptidases and arguments could be made that stabilizing the amino terminal end of a peptide would be more effective at preventing degradation by peptidases than stabilizing the carboxy terminal end of the peptide. To test whether peptides could be stabilized by fusing the amino terminus of the peptides to the carboxy terminus of the Rop protein, the p(N)Rop- fusion vector shown in Figure 3, which is a derivative of pLAC11, was constructed as described in Materials and Methods. In order to isolate potential inhibitor peptides which were protected at their amino-terminal end, a totally randomized oligonucleotide library that encoded up to 20 amino acid peptides was cloned into the p(N)Rop- fusion vector as shown in Figure 4 and transformed into *E. coli* under repressed conditions. 6000 of these Rop fusion peptides were screened using the grid-patching technique and 14 two day inhibitors were isolated. As with the Rop fusion peptides which were isolated using the p-Rop(C) vector, we found that most of the inhibitor peptides isolated using the p(N)Rop- vector were inhibitory on rich media as well as minimal media. The inhibitors were verified as described above and subjected to growth rate analysis using p(N)Rop- as a control in order to determine their potency. As indicated in Table 2, all of the inhibitors inhibited bacterial growth at levels greater than 90%.

Isolation and characterization of anchorless inhibitor peptides that are protected at both their amino terminal and carboxy terminal ends: The results of the Rop-peptide fusion studies suggested that

peptides could be stabilized by protecting either their amino or carboxy terminal ends. We therefore decided to test whether anchorless peptides could be generated which were protected by two proline residues placed at both the amino terminal and carboxy terminal ends of the peptide, based on three independent lines of evidence. First, in our previous studies with anchorless inhibitor peptides, we had noticed that 40% of them contained one or more prolines near or at their carboxy terminal ends (24). Second, it has been speculated that peptidases have a hard time degrading proline residues (23, 27). Third, several naturally occurring bioactive peptides, PR-39, Bac5, and Bac7 have been identified which are proline rich (1, 10). In order to isolate potential inhibitor peptides which were protected at both ends by two proline residues, a totally randomized oligonucleotide library that encoded 20 amino acid peptides was cloned into the pLAC11 expression vector (25) as shown in Figure 5 and transformed into *E. coli* under repressed conditions. 5000 peptides were screened using the grid-patching technique and 22 two day inhibitors were isolated. The inhibitors were verified as already described for the Rop-peptide fusion studies and subjected to growth rate analysis using pLAC11 as a control in order to determine their potency. As indicated in Table 3, all of the inhibitors inhibited bacterial growth at levels greater than 90%.

DISCUSSION

While there appears to be great promise for the design and use of synthetic inhibitor peptides as novel therapeutic agents in medicine, there are still problems that must be overcome. Perhaps the most severe problem is the fact that many of the synthetic peptide drugs have been found to be unstable and are degraded in the host cell (3, 8, 26). Approaches to solve this problem have centered on developing delivery systems which will enable the peptide drugs to avoid degradation by the numerous peptidases that are present in host cells or else preventing the action of the peptidases by cyclization or employing D-amino acids or modified amino acids instead of naturally occurring L-amino acids in the peptides. We have taken an alternative approach to this problem and are trying to find ways by which the peptides can be stabilized through the implementation of structural motifs in order to prevent the action of peptidases.

We have argued that bioactive peptides need to possess some sort of stabilizing motif if they are to be functional based on our analysis of novel bioactive peptides that were isolated which could inhibit the growth of *E. coli* bacteria as well as what has been learned about the structure of naturally occurring bioactive peptides (24). In this study, summarized in Table 4, we have investigated ways by which bioactive peptides can be stabilized to prevent the action of amino and carboxypeptidases. In our previous study of naked anchorless bioactive peptides, we found that only 1 out of 20,000 peptides proved to be potent inhibitors of *E. coli* bacteria. In this study, we found that if either the amino or carboxy terminal end of the peptide was protected by fusing the peptide to the very stable Rop protein anchor, the frequency at which we could isolate potent inhibitors increased by 37-fold.

Based on the fact that many more aminopeptidases have been identified than carboxypeptidaess in both prokaryotic and eukaryotic cells (2, 5, 15), one might have assumed that stabilizing the amino terminal end of the peptide would have been more effective at preventing the action of exopeptidases than stabilizing the carboxy end of the peptides. We find that stabilizing either end of the peptide causes about the same effect. There are two simple explanations for our findings. Either the carboxypeptidases are more potent than the aminopeptidases, or else there are simply more carboxypeptidases that have yet to be identified.

Because the results of our Rop-peptide fusion studies suggested that stabilizing the ends of inhibitor peptides could drastically increase the frequency at which potent inhibitors could be isolated,

we wanted to investigate whether there was some way by which we could stabilize naked anchorless peptides without the use of a fusion protein. In our previous studies we had noticed that many of the anchorless inhibitor peptides that we had isolated appeared to be potentially stabilized by the presence of one or two proline residues near or at the carboxy terminal end of the peptide. Since it has been suggested that proline residues may make it harder for peptidases to act on a peptide (23, 27) and there are some examples of proline rich bioactive peptides (1, 10), we decided to investigate whether placing two proline residues at both ends of a peptide would result in more stable peptides. As shown in Table 4, the addition of two prolines to both ends of the peptide caused the frequency at which potent inhibitor peptides could be isolated to increase by 88-fold over that of the anchorless peptides that we had originally characterized. Interestingly, this increase is roughly twice that of what can be achieved by protecting only the amino or carboxy terminal end of the peptide.

We believe that our findings can be directly implemented in order to design more effective peptide drugs that are not as subject to degradation by peptidases. Because the amino and carboxypeptidases that have been characterized in prokaryotic and eukaryotic systems appear to function quite similarly (15, 17), placing two prolines at the beginning and end of designer peptide drugs should prevent the action of exopeptidases in the host cell. In future studies, we plan to investigate whether the placement of two prolines at only the amino terminal or carboxy terminal ends of the peptides is just as effective at generating potent inhibitor peptides, as well as testing whether a single proline will work just as well as two prolines. Our laboratory is also interested in studying whether other motifs can be used to stabilize designer inhibitor peptides and we have found that if peptide libraries are synthesized using a helix-generating motif that the frequency at which inhibitor peptides can be isolated is 10-fold greater than the frequency at which inhibitor peptides can be generated using a completely randomized library (Warren and Altman, unpublished observations).

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FIGURE LEGENDS:

Figure 1: Map of the p-Rop(C) fusion vector. The unique restriction sites and the base pair at which they cut are indicated. Key sites of interest are also shown; Rop (7-198), ori (627-1214), Amp (2245-1385), lacPO (2500-2612).

Figure 2: Scheme for generating the randomized 20 amino acid peptide library where the carboxy terminal end of the peptides are fused to the amino terminal end of the Rop protein. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, are used. Because of the way the oligonucleotide library has been engineered, either orientation of the incoming digested ds DNA fragment results in a fusion product.

Figure 3: Map of the p(N)Rop- fusion vector. The unique restriction sites and the base pair at which they cut are indicated. Key sites of interest are also shown; Rop (7-204), ori (266-853), Amp (1024-1884), lacPO (2139-2251).

Figure 4: Scheme for generating the randomized 20 amino acid peptide library where the amino terminal end of the peptides are fused to the carboxy terminal end of the Rop protein. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, are used. Because of the way the oligonucleotide library has been engineered, either orientation of the incoming digested ds DNA fragment results in a fusion product.

Figure 5: Scheme for generating the randomized 20 amino acid peptide library where the peptides are protected at both the amino terminal and carboxy terminal ends by two proline residues. The complementary strand of the 93 base randomized oligonucleotide is generated by filling-in with Klenow using the 24 base oligonucleotide primer. The resulting ds DNA is digested with Bgl II and EcoR I and then ligated into the pLAC11 expression vector which has also been digested with the same two restrcition enzymes. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, are used.

Table 3: Inhibitory effects of peptide inhibitors that are stabilized by two proline residues at both the amino and carboxy terminal ends of the peptide

Inhibitor	% inhibition in minimal media	% inhibition in rich media
pPro1	99.0	87.0
pPro2	98.2	83.8
pPro3	95.6	82.6
pPro4	98.6	84.7
pPro5	96.2	84.4
pPro6	97.0	81.8
pPro7	97.6	80.6
pPro8	97.6	89.0
pPro9	99.0	83.6
pPro10	93.1	69.8

The inhibitory effects were determined as described in the text using pLAC11 as a control. The data is the average of duplicate experiments.

Table 4: Summary of the frequency at which the different types of inhibitor peptides can be isolated

Type of inhibitor peptide	Frequency at which a two day inhibitor peptide can be isolated	Reference
anchorless	1 in 20,000	Example 2
protected at the C-terminal end via Rop	1 in 625	This study
protected at the N-terminal end via Rop	1 in 429	This study
protected at both the C-terminal and N-terminal end via two prolines	1 in 227	This study